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System-wide molecular evidence for phenotypic buffering in *Arabidopsis*

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Supplementary Tables:

Supplementary Table 1: All 139 phenotypic (morphological, physiological, pathological) and 98 biochemical traits collected from the literature.

No.	Trait ID	Trait Category	Description	Ref
1	LDVer-FT	flowering time	Flowering time under long daylight conditions and after vernalization treatment	1
2	LDVer-RLN	rosette number	Rosette leaf number under long daylight conditions and after vernalization treatment	1
3	LDVer-CLN	cauline number	Cauline leaf number under long daylight conditions and after vernalization treatment	1
4	LDVer-TLN	leaf number	Total leaf number under long daylight conditions and after vernalization treatment	1
5	LD-FT	flowering time	Flowering time under long daylight conditions (without vernalization)	1
6	LD-RLN	rosette number	Rosette leaf number under long daylight conditions (without vernalization)	1
7	LD-CLN	cauline number	Cauline leaf number under long daylight conditions (without vernalization)	1
8	LD-TLN	leaf number	Total leaf number under long daylight conditions (without vernalization)	1
9	SD-FT	flowering time	Flowering time under short daylight conditions	1
10	SD-RLN	rosette number	Rosette leaf number under short daylight conditions	1
11	SD-CLN	cauline number	Cauline leaf number under short daylight conditions	1
12	SD-TLN	leaf number	Total leaf number under short daylight conditions	1
13	SL	seed length	Seed length (in mm)	2
14	SW	seed weight	Seed weight (mg per 100 seeds)	2
15	FL	fruit length	Fruit length (in mm)	2
16	OL	ovary length	Ovary length (in mm)	2
17	ON	ovule number	Ovule number per fruit	2
18	SN	seed number	Seed number per fruit	2

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19	Unf	ovule number	Unfertilized ovule number per fruit	2
20	TLN	leaf number	Total leaf number	2
21	LLL	leaf length	Largest leaf length (in mm)	2
22	PH	plant height	Plant height (in cm)	2
23	SSN	shoot number	Side shoot number	2
24	FN	fruit number	Fruit number in the main stem	2
25	Sw	seed weight	Seed weight of 100 seeds (in milligram)	3
26	Sucrose	sugar content	Mean sucrose content in micrograms per seed milligram	3
27	Raffinose	sugar content	Mean raffinose content in micrograms per seed milligram	3
28	Stachyose	sugar content	Mean stachyose content in micrograms per seed milligram	3
29	CD0d	germination	Mean germination percentage after 0 days of CD treatment	3
30	CD4d	germination	Mean germination percentage after 4 days of CD treatment	3
31	CD4/0d	germination	Fraction of the mean germination percentage after 4 days of CD treatment in relation to the mean germination percentage after 0 days of CD treatment	3
32	pCD0d	germination	Probit of the mean germination percentage after 0 days CD treatment	3
33	pCD4d	germination	Probit of the mean germination percentage after 4 days CD treatment	3
34	pCD4/0d	germination	Probit of the fraction of mean germination percentage after 4 days of CD treatment in relation to the mean germination percentage after 0 days of CD treatment	3
35	germ4y	germination	Mean germination percentage after 4 years of seed storage	3
36	pgerm4y	germination	Probit of germination percentage after 4 years of seed storage	3
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43	Brz	hypocotyl length	Mean hypocotyl length of seedlings grown in darkness and with brassinazole	4
44	sgewicht	seed weight	Seed weight (in mg)	5
45	sphytate	phytate/phosphate content	Phytate content in milligrams per gram seed	5
46	sphosphate	phytate/phosphate content	Phosphate content in milligrams per gram seed	5
47	lPhosphate	phytate/phosphate content	Phosphate content in micrograms per gram leaf (fresh weight)	5
48	lPhytate	phytate/phosphate content	Phytate content in micrograms per gram leaf (fresh weight)	5
49	ldtof	flowering time	Time to flower (bolting) under long daylight condition	6
50	ldrln	rosette number	Rosette leaf number under long daylight condition	6
51	ldrd	rosette dismeter	Rosette diameter under long daylight condition	6
52	ldph	plant height	Plant height (cm) under long daylight condition	6
53	ldtm	maturation time	Time to maturate under long daylight condition	6
54	ldlr	reproductive length	Length of reproductive phase under long daylight condition	6
55	ldtea	axil number	Total number elongated axils under long daylight condition	6
56	ldnec	cauline number	Cauline leaves at maturity under long daylight condition	6
57	ldnmf	flower number	Number of main axis fruits/flowers at maturity under long daylight condition	6

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59	ldtaf	flower number	Number of axilliar fruits/flowers at maturity under long daylight condition	6
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61	ldtf	flower number	Total number of flowers under long daylight condition	6
62	sdtof	flowering time	Time to flower (bolting) under short daylight condition	7
63	sdrln	rosette number	Rosette Leaf number under short daylight condition	7
64	sdrd	rosette diameter	Rosette diameter under short daylight condition	7
65	sdph	plant height	Plant height (cm) under short daylight condition	7
66	sdtm	maturation time	Time to maturate under short daylight condition	7
67	sdlr	reproductive length	Length of reproductive phase under short daylight condition	7
68	sdtea	axil number	Total number elongated axils under short daylight condition	7
69	sdnec	cauline number	Cauline leaves at maturity under short daylight condition	7
70	sdnmf	flower number	Number of main axis fruits/flowers at maturity under short daylight condition	7
71	sdtim	inflorescence number	Total number inflorescence meristems under short daylight condition	7
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Supplementary Information

Supplementary Figures

Supplementary Figure 1: Hotspots and other QTLs

The distribution of the number of traits that map to each of the six hotspots (pink and blue balls) and/or to the other genome regions (grey ball) is shown. A blue ball indicates that the hotspot was not significant, while a pink ball indicates the hotspot was significant at the given level. Dark lines between two balls report the number of traits that mapped to the two corresponding hotspot loci (in which case the trait has two or more QTLs). Grey lines report the number of traits that mapped to one or more hotspots and to another genome region.

Most transcripts and proteins map to one QTL only. Many metabolites map to two QTLs and patterns of connectivity emerge, e.g. many metabolites possess one hotspot mQTL and one other non-hotspot mQTL, which suggests that the whole metabolome has a correlated structure rather than that most hotspot mQTLs were observed for a selected set of metabolites only. Metabolic and phenotypic traits can also map to multiple hotspots, e.g. 33 phenotypic traits map to CRY2 and one of the other hotspots, which suggests that different subordinate network components became connected.

Together, these six hot spots influence 16%, 25%, 55% and 77% of 4,832, 253, 7,158 and 116 transcript, protein, metabolite and phenotypic traits with QTLs, respectively, when a window of 5 cM around the hot spot is used to account for imperfect mapping resolution in the QTL analysis; 13%, 15%, 50% and 69% when using a 2 cM window.

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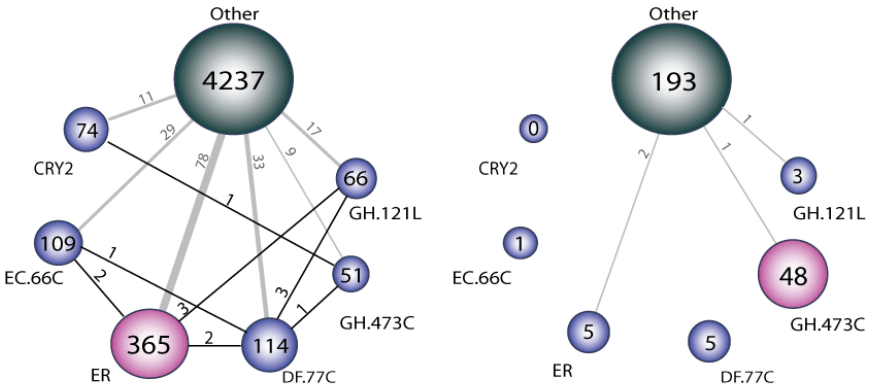
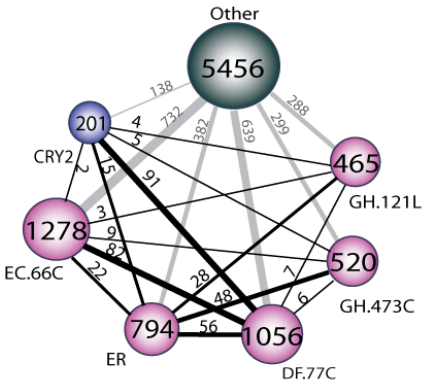
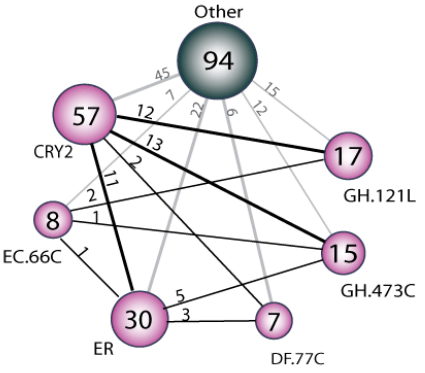


Transcript

Protein



Metabolite



Phenotype

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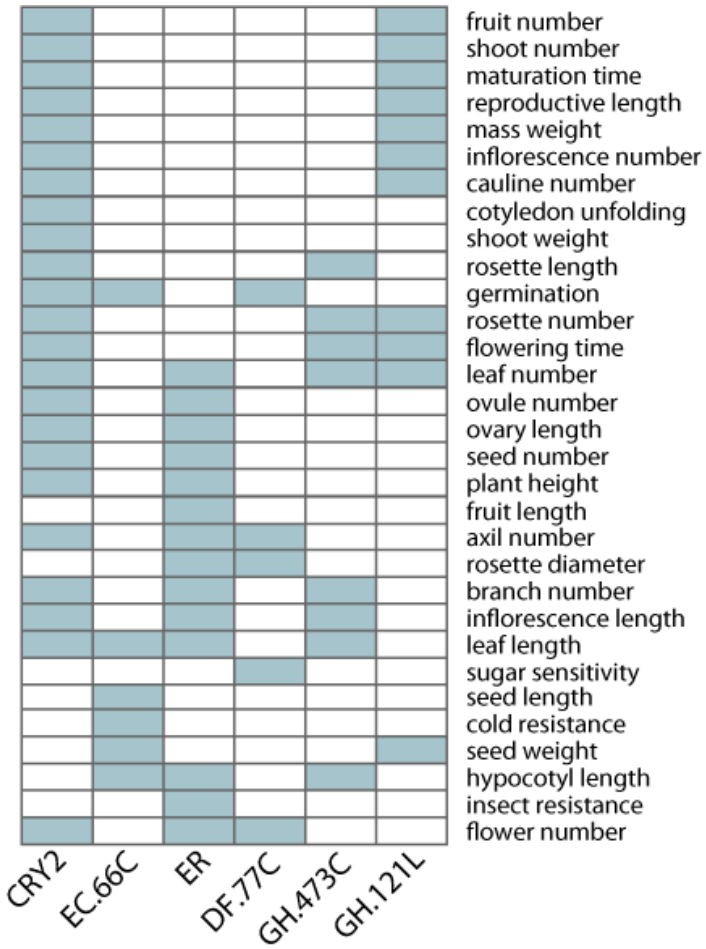
Supplementary Information

Supplementary Figure 2: Phenotypic trait categories

The phenotypic traits collected from the literature (**Supplementary Table 1**) could be partly redundant, because occasionally very similar traits have been measured multiple times, although under different conditions, at different stages, or by different groups. We grouped the 139 traits into 35 distinct phenotypic trait categories. 33 phenotypic trait categories have traits with QTLs (116 traits in total). 31 phenotypic trait categories (94%) have at least one trait with a QTL in a window of 5 cM around a hotspot (blue cells). See **Supplementary Fig. 3** for further analysis.



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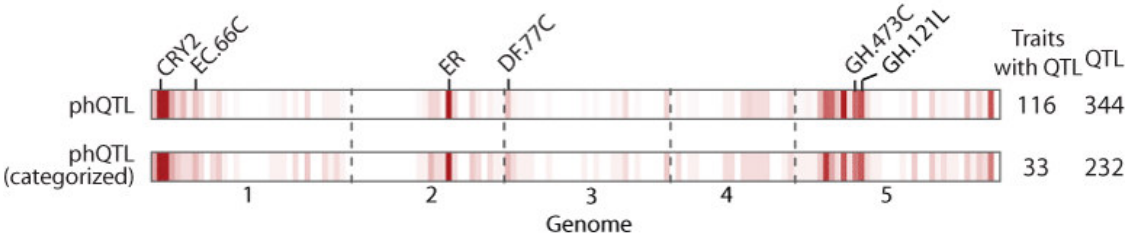
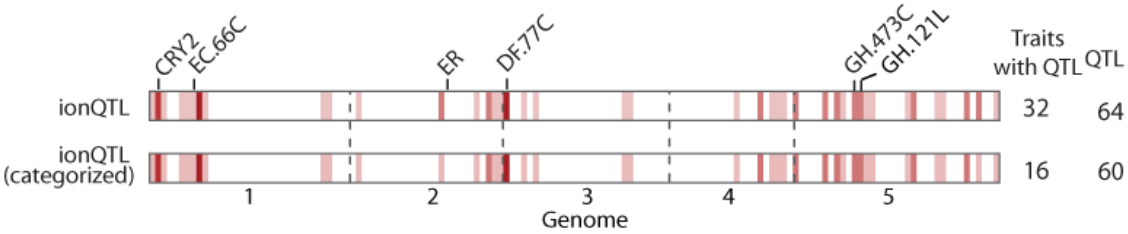
Supplementary Information

Supplementary Figure 3: Further analysis of trait data collected from literature

(a) Phenotypic traits. The 139 phenotypic traits (**Supplementary Table 1**) were grouped into 35 distinct categories (**Supplementary Figure 2**). We here show the heat maps with and without categorization. If multiple traits belong to the same category and map to the same locus, we counted it as a single QTL for this category. Clearly, the two phQTL distributions are almost identical and not affected much by potential redundancy within phenotypic trait categories. Consequently, the system-wide effects of the six hotspots are also almost identical. The number of phenotypic traits with a hotspot QTL is: 89 out of 116 traits with a QTL (77%). The number of trait categories with a hotspot QTL is: 31 out of 33 categories with a QTL (94%).



(b) Biochemical traits. The 98 biochemical traits collected from the literature predominantly consisted of 49 traits for 19 distinct ions, and 28 traits for 3 distinct proteins only. Therefore we here show the heat maps with and without categorization for the larger collection of ions only. The number of ion traits with a hotspot QTL, is: 22 out of 32 traits with a QTL (69%). The number of ion trait categories with a hotspot QTL is: 14 out of 16 categories with a QTL (88%), using a window of 5 cM around the hotspot to account for imperfect mapping resolution in QTL analysis.



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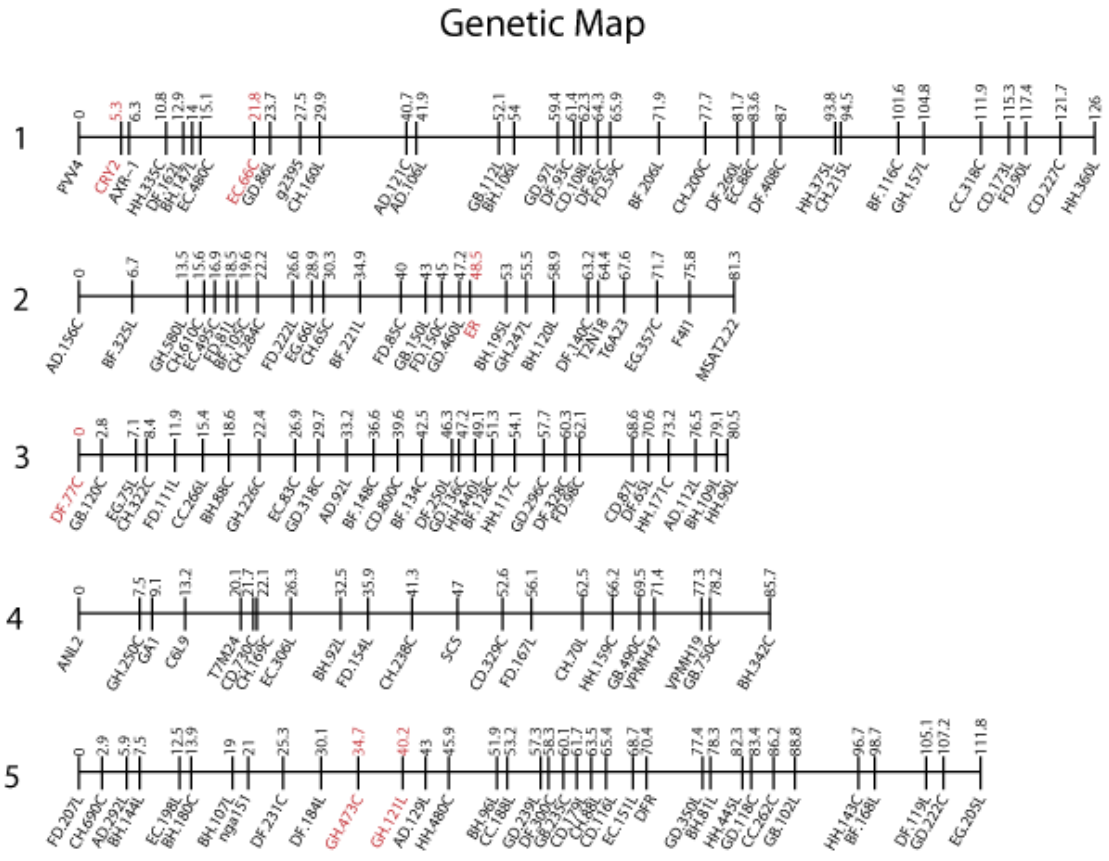
Supplementary Information

Supplementary Figure 4: The genetic map

The names and the map positions (in cM) of the 144 markers are shown. The six hotspot markers are highlighted in red.



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Supplementary Information

Supplementary Figure 5: Traits mapping to QTL hotspots: QTL likelihood profiles.

We detected six QTL hotspots on the genome. QTL likelihood profiles of individual molecular and phenotypic traits mapping to these hotspots can be accessed via six different figures: **Supplementary Fig. 5a** for traits that map to hotspot 1, **Supplementary Fig. 5b** for traits that map to hotspot 2, and so on. Traits that map to two or more hotspots will appear in two or more figures.

The first row in **Supplementary Fig. 5a** shows a heat map of the average QTL profile for all traits that map to hotspot 1 (located at marker 2) and that also map to marker 143 (there are 144 markers in total, but no traits map to marker 2 and marker 144). The second row refers to all traits that map to hotspot 1 and to marker 142, and so on (in this and in the five other figures). The numbers at the right-hand side of the heat map indicate the number of transcript-, protein-, metabolite- and phenotypic traits in the row. Chromosomes are indicated and the red dashed lines are the chromosome borders.

The heat maps are hyperlinked to the QTL likelihood profiles of the traits on http://gbic.biol.rug.nl/supplementary/2008/phenotypic_buffering/supFig5.htm. Clicking on a row of the heat map will open a new window showing the QTL likelihood profiles of individual traits.

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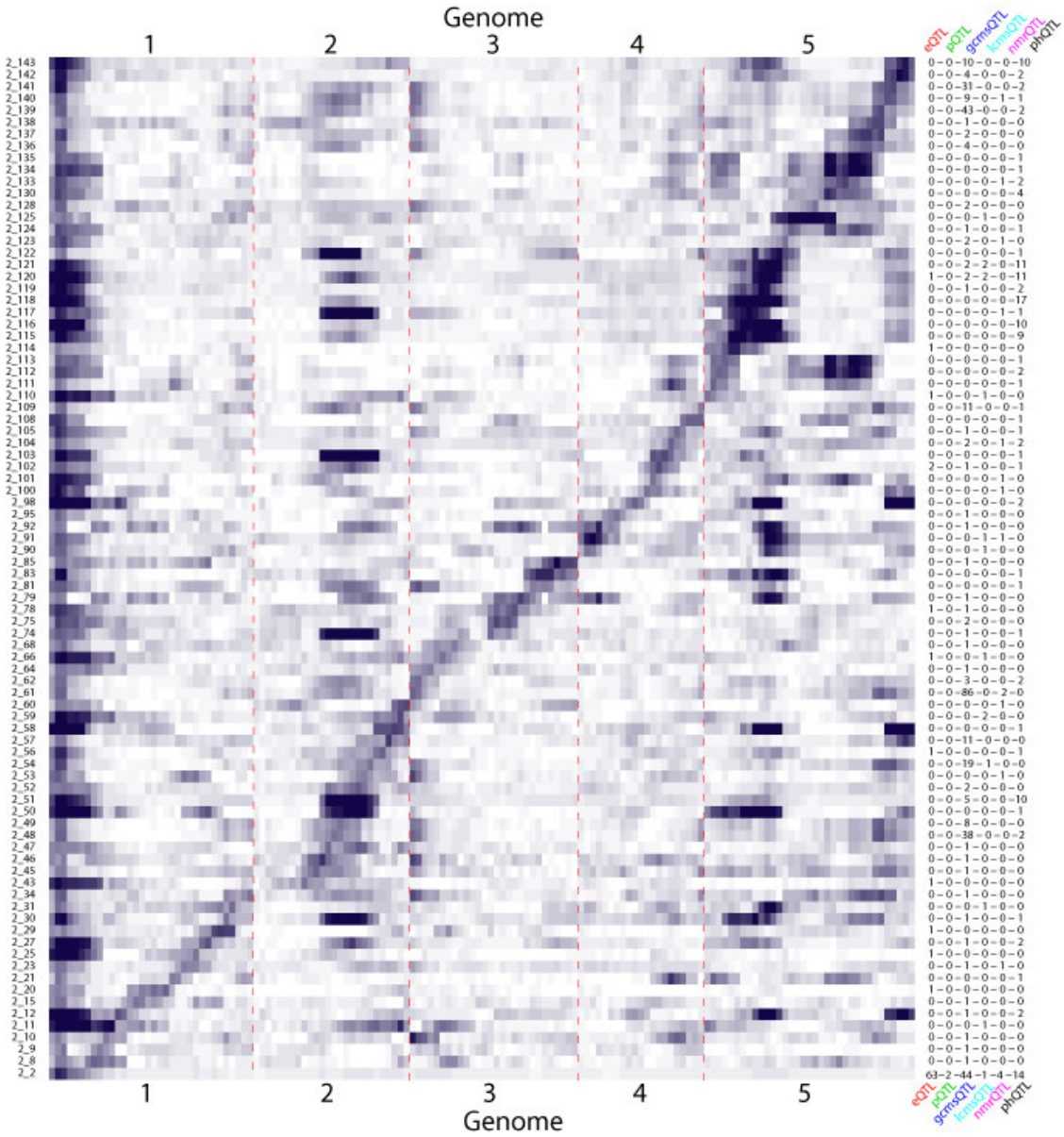
Supplementary Information

Fig 5a. Traits mapping to hotspot CRY2.



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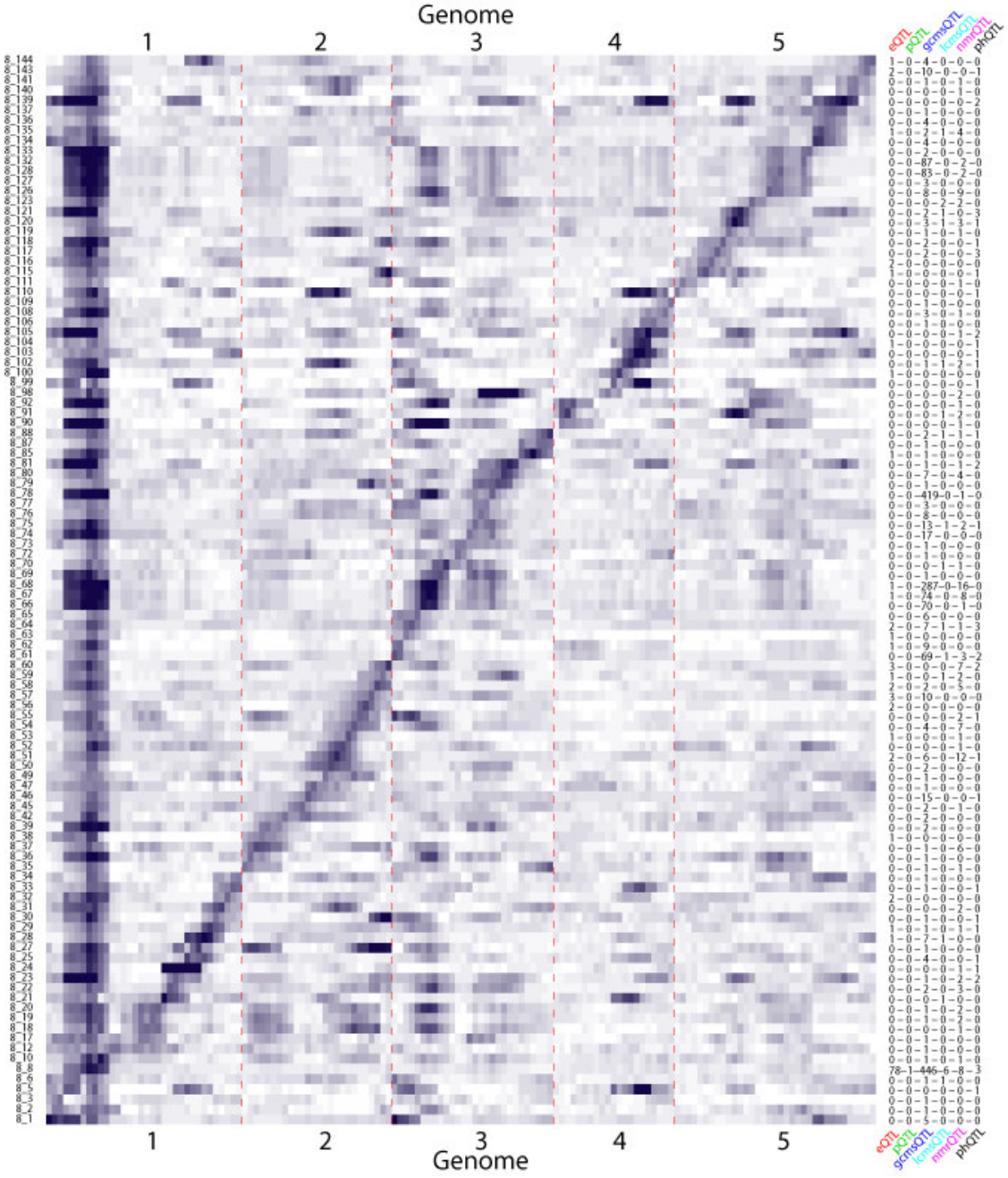
Supplementary Information

Fig 5b. Traits mapping to hotspot EC.66C



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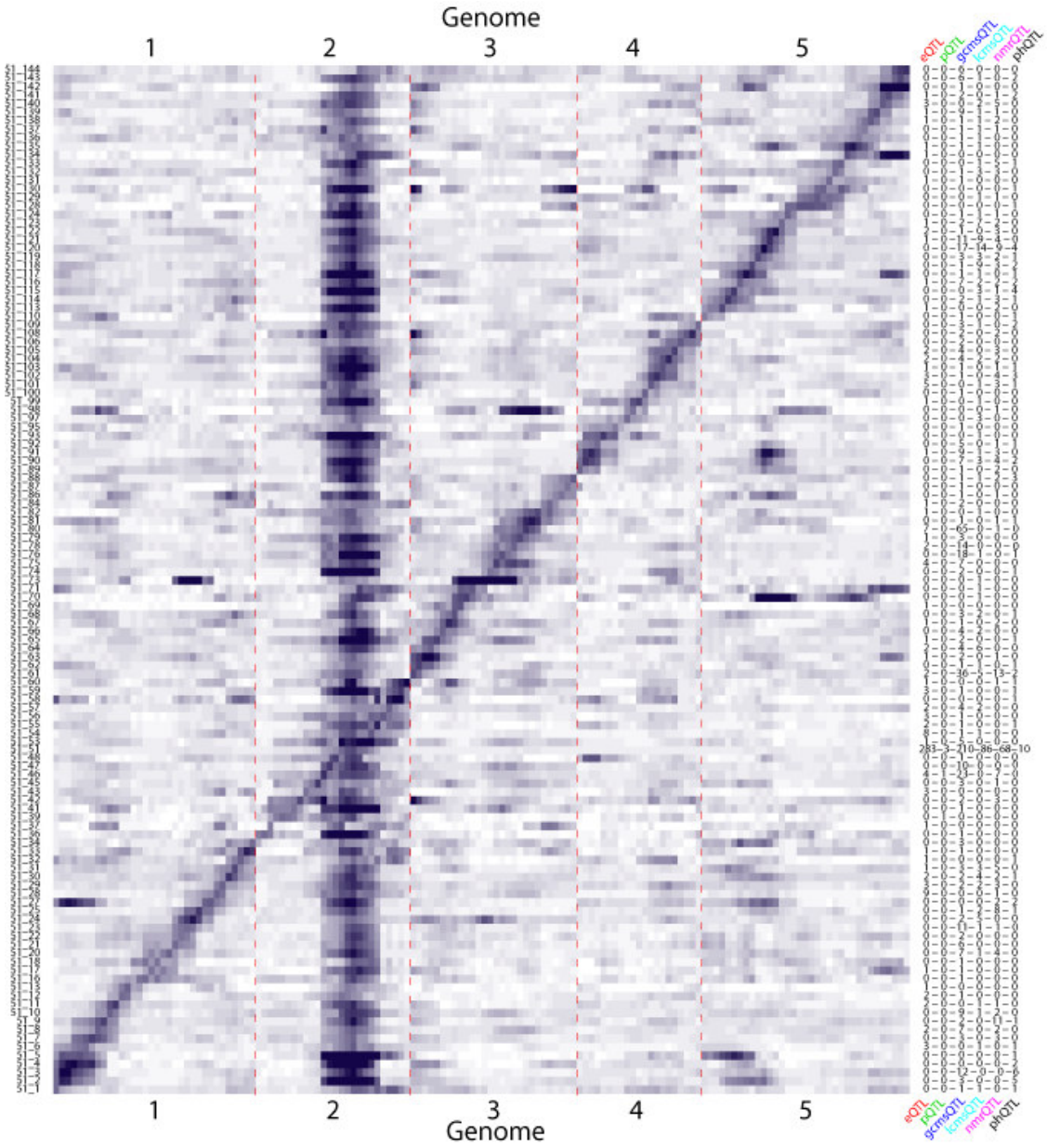
Supplementary Information

Fig. 5c. Traits mapping to hotspot ER



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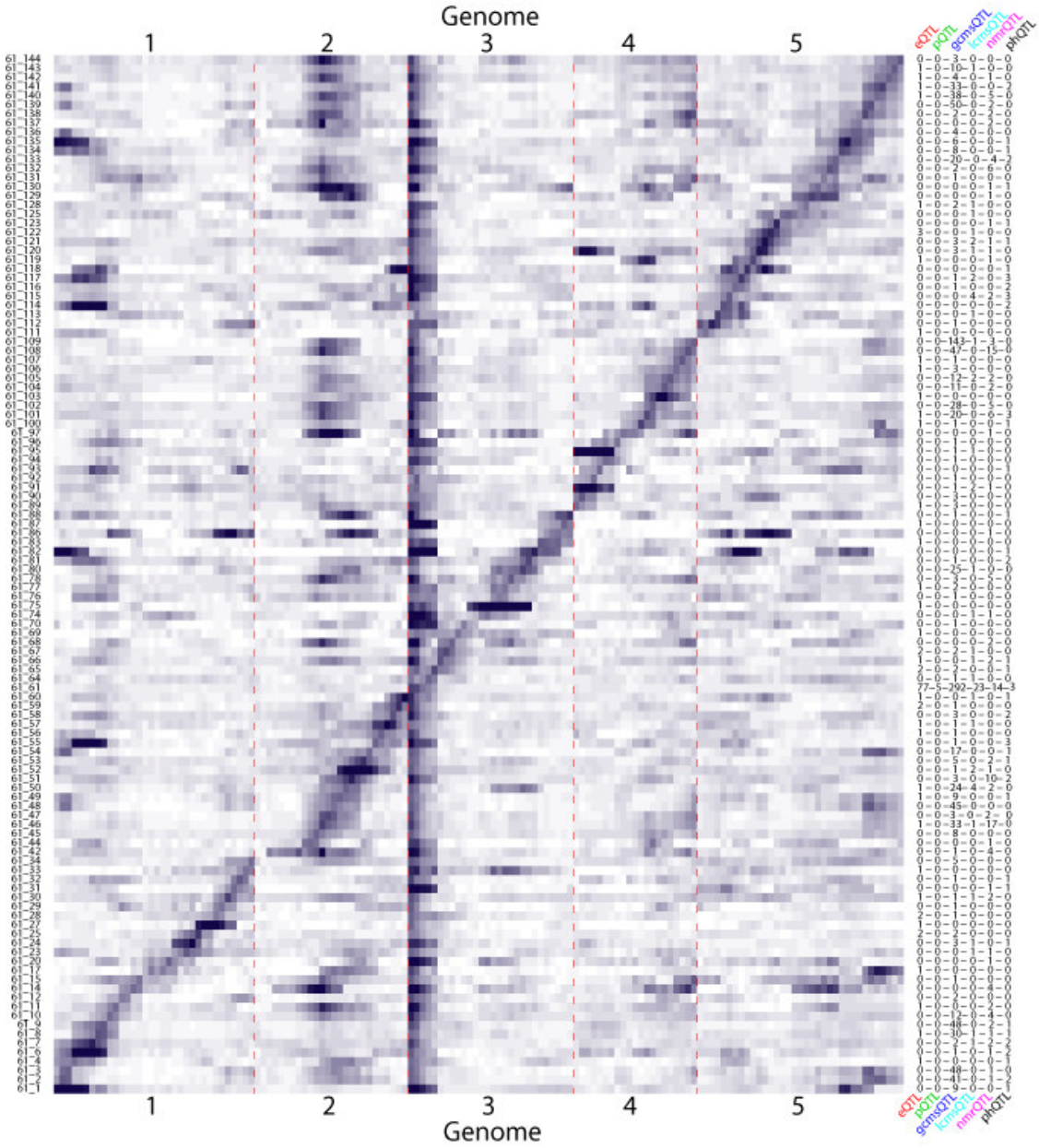
Supplementary Information

Fig. 5d. Traits mapping to hotspot DF.77C



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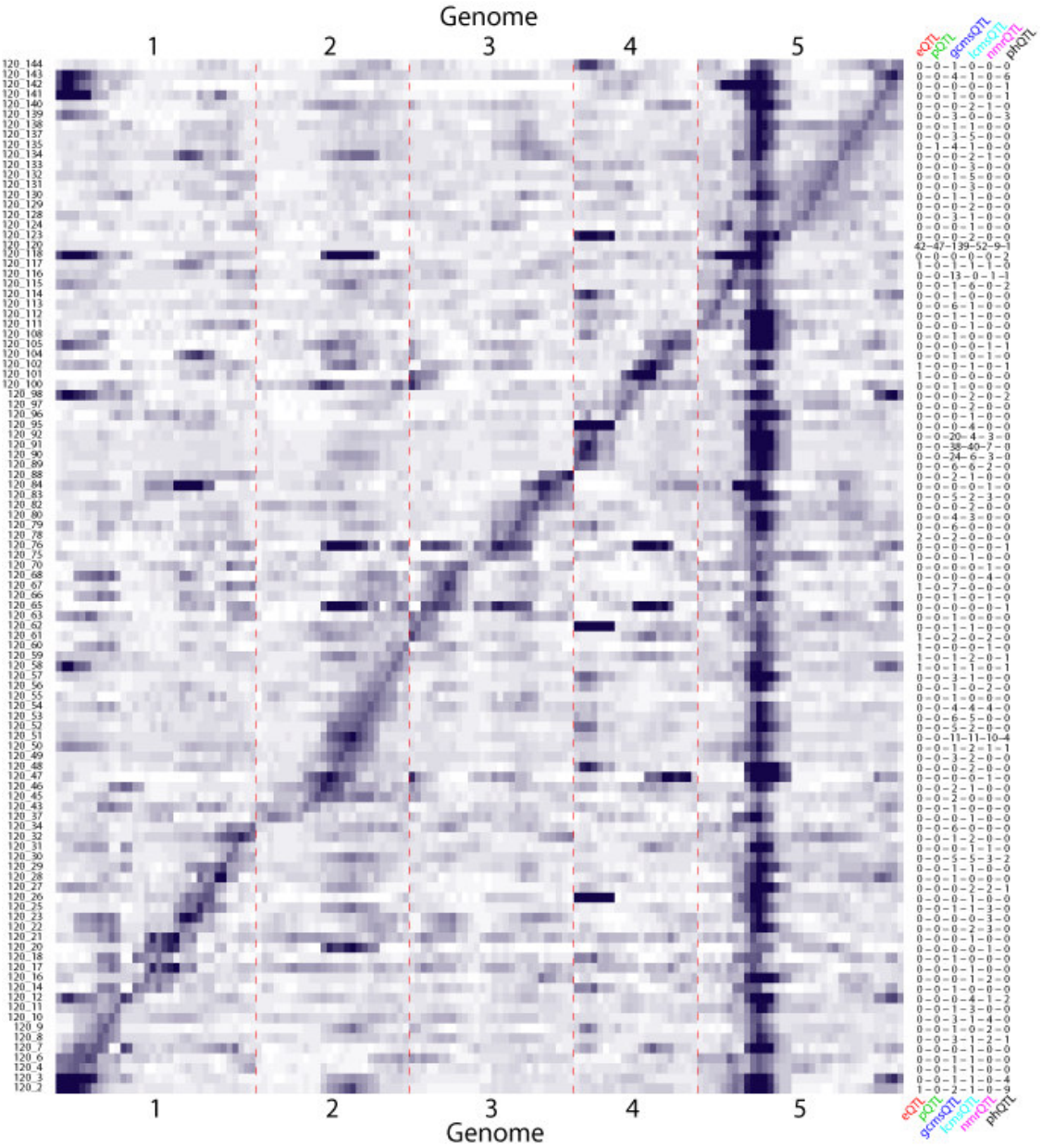
Supplementary Information

Fig. 5e. Traits mapping to hotspot GH.473C



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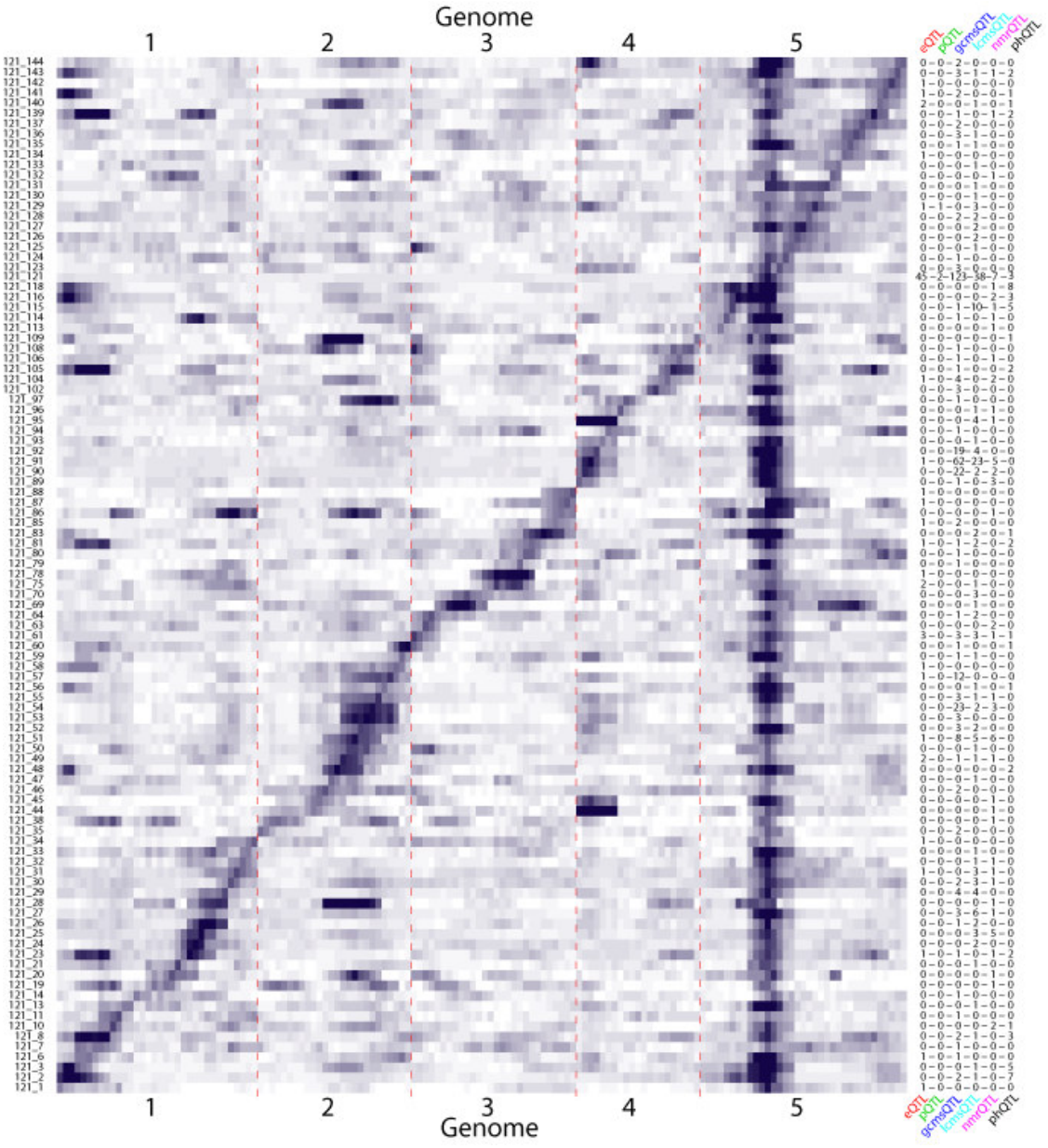
Supplementary Information

Fig. 5f. Traits mapping to hotspot GH.121L



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Supplementary Information

Supplementary Methods

1. Plant growth conditions and harvesting

Seeds of RILs were sown on 10 ml ½ MS agar (2%) in Ø 6 cm Petri dishes. Per line five replicate dishes were sown on five consecutive days with a density of a few hundred seeds per Petri dish. Petri dishes were placed in a cold room at 4°C for 7 days in the dark to promote uniform germination. Subsequently dishes were randomly placed in five blocks in a climate chamber, where each block contained one replicate dish of each line. Growing conditions were 16 h light (30 $W.m^{-2}$) at 20°C, 8 h dark at 15°C and 75% relative humidity. After 6 days the lids of the Petri dishes were removed to ensure seedlings were free of condensed water on the day of harvesting. On day 7, seedlings were harvested by submerging the complete Petri dish briefly in liquid nitrogen and scraping off the aerial parts with a razor blade. Harvesting started 7 hours into the light period and all lines were harvested in random order within 2 hours. Per line, plant material from two dishes was harvested to make one replicate sample and from the other three dishes to make the second. Plant material was ground in liquid nitrogen and stored at –80°C until further processing (full details see ref[1]).

2. New molecular data we have generated on all RILs

2.1 2D-PAGE

For protein extraction, (liquid nitrogen) frozen Arabidopsis seedlings were ground with pestle and mortar. To 100 mg material, weighed in a 2 ml screw-cap tube, we added 1.5 ml of 10%TCA, 0.3% DTT (w/v) in acetone. Samples were homogenized in the FastPrep® system (Qbiogene, Irvine, USA) for 45 seconds at a speed of 6.5 rpm and incubated at –20ºC for at least one hour, with occasional vortexing. The homogenate was centrifuged for 30 minutes at 20,800 g 4ºC). The supernatant was decanted and the pellet was washed twice with 1.5ml acetone (10 mM DTT). After air-drying, the pellet was solubilized in 200 µl TUCD buffer (6 M urea, 2 M thio-urea, 2% (w/v) CHAPS and 30 mM Tris-HCl pH8,5). Protein amount was measured using the RC/DC assay (Bio-Rad) using BSA as reference. Some sample extracts with low protein concentration were concentrated and cleaned using the clean-up kit (GE Healthcare, manufacturer's protocol) and dissolved TUCD buffer to a final protein concentration of 5 ug/ul.

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Proteins were labeled using the fluorescent CyDyes from the Difference Gel Electrophoresis (DIGE®) technology (GE Healthcare, Sweden) according to the manufacturer's protocol. For each sample, 50 µg of protein was labeled with 0.4 nmol Cy3 or Cy5. For internal standard, a reference sample was prepared by mixing equal protein amounts of all samples (131 in total). This mixture was labeled with Cy2. Each 2D-gel contains one sample labeled with Cy3, one labeled with Cy5 and the internal standard labeled with Cy2.

The first-dimension isoelectric focusing was performed using 24 cm immobilized pH gradient strips (GE Healthcare) with a linear pH range of 4 to 7 in an Ettan IPGPhor isoelectric focusing system. Cydye 2, 3 & 5 labeled samples (total of 150 µg protein) were diluted in 0.5% IPG buffer (pH 4-7 and pH 3-10, 1:1) and TUCD buffer to a volume of 450 µl and loaded into the strips by rehydration. The focusing was run overnight at 20ºC with the following settings: 30 V for 1 h, 500 V for 1 h, from 500 V to 1000 V in 6 h, from 1000 V to 8000 V in 3 h, and finally 8000 V until a total of 60,000 Vhour was reached. After IEF the strips were equilibrated at room temperature in equilibration buffer (6 M urea, 50 mM Tris-HCl pH 8.8, 30% (v/v) glycerol, 2% (w/v) SDS containing 1% (w/v) DTT for 15 minutes and after that in the same buffer containing 2.5% (w/v) iodoacetamide for 10 minutes. The second dimension was run in the ISO-DALT system (GE healthcare) on 11% SDS-PAGE slab gels. Electrophoresis was performed at 30 V for 1 hour followed by 90 V overnight at 10ºC. The gels were scanned remaining in the glass plate, using the Ettan DIGE Imager (GE Healthcare, using the manufacturer's setting for CyDye detection).

Gel images were analyzed with the Decyder software V6.5 (GE Healthcare). Automated spot detection was performed (estimated number of spots 2500). The detected spots were then filtered based on spot area (>270), spot volume (>$1.1e10^4$), peak height (>80), peak slope (< 0.75) to exclude background noise and dust particles. The internal standard in each gel was used to automatically match all images to the reference (the gel with the most detected spots). The matching was checked and corrected by hand with the help of land-marking clear spots that were visible in all images and rematching the dataset.

2.2 GC-TOF-MS

Frozen and ground samples of approximately 50 mg fresh weight were weighed accurately in a 2 ml Eppendorf vial with punctured lid, and 1.4 ml cold methanol and

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ribitol as internal standard were added. Samples were extracted for 20 min in a shaking water bath at 70°C. After centrifuging at 21000 g for 5 min, 500 µL of the supernatant was transferred to a new 2 ml Eppendorf vial. A two-phase extraction method was used to separate polar and apolar compounds by adding 500 µl water and 700 µl chloroform[2]. After vortexing and centrifugation at 21000 g for 5 min, 200 µL of the polar phase was dried under vacuum. The dried extracts were derivatized by methoximation and trimethylsilylation essentially as described[2] using a CombiPal robot for on-line derivatization. Octadecane was added to the o-methylhydroxiaminehydrochloride in pyridine to check for accuracy of the pipetting of the robot.

Samples were injected with an Optic3 injector (ATAS) at 70°C with a gradient of 6°C/sec to 240°C using a split flow of 10 ml and a column flow of 2 ml in a GC6890N gas chromatograph (Agilent Technologies) on a ZB50 capillary column (30 m x 0.32 mm i.d., 0.25 µm DF; Phenomenex) with a column temperature of 70°C for 2 minutes and a gradient of 10°C/min to 310°C and a final time of 3 min. The GC was coupled to a Pegasus III time-of-flight mass spectrometer (LECO) and compounds were detected at a scanning rate of 20 spectra per second (mass 50-600). Metalign™ software (www.metalign.nl) was used to extract all mass signals detected and to align these signals across the samples[3].

2.3 ^{1}H NMR

NMR sample preparation was carried out according to the procedures described in ref[4, 5]. NMR extractions were performed for three technical replicates of each biological sample. Freeze-dried leaf samples (15 mg) were extracted at 50^{0}C for 10 min with 80:20 D_2O:CD_3OD (1 ml) containing 0.05% w/v d_4-TSP. After cooling and centrifugation (15 minutes), the supernatant was transferred to a clean Eppendorf tube and heated to 90°C for 2 min. Samples were then cooled to 8°C for 30 min before centrifugation (10 min). 750 µl of the supernatant was transferred to a 5 mm NMR tube for ^{1}H NMR analysis. NMR extractions were performed for three replicates of each biological sample.

^{1}H NMR spectra were acquired under automation at 300ºK on an Avance Spectrometer (Bruker, Coventry, UK) operating at 600.0528 MHz, equipped with a 5 mm Selective Inverse Probe. Spectra were collected using a water suppression pulse sequence with a relaxation delay of 5 s. Each spectrum was acquired using 128 scans of 64,000 data points with a spectral width of 7309.99 Hz. Spectra were automatically Fourier transformed using an exponential window with a line broadening value of 0.5 Hz.

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Phasing and baseline correction were carried out within the instrument software. ^{1}H chemical shifts were referenced to d_4-TSP at δ0.00.

^{1}H NMR spectra were automatically reduced, using Amix (Analysis of MIXtures software, Bruker Biospin, Coventry, UK), to ASCII files containing integrated regions or "buckets" of equal width (0.01 ppm). Spectral intensities were scaled to the d_4-TSP region (δ0.05 to –0.05). The ASCII file was imported into Excel for the addition of sample details. The regions for unsuppressed water (δ4.865-4.775), d_4-MeOH (δ3.335-3.285) and d_4-TSP (δ0.05 to –0.05) were removed.

Identification of individual metabolites was achieved by comparing to authentic standards whose spectra were collected under the same experimental conditions.

3. Molecular data we have previously generated on all RILs

3.1 mRNA-microarrays

Total RNA of each line was isolated from two biological replicates by using phenol-chloroform extraction. Genome-wide gene expression analysis was carried out using two-color microarrays provide by the Galbraith laboratory (University of Arizona, Tucson, AZ) with Qiagen Operon (Valencia, CA/Alameda, CA) Arabidopsis genome oligo set Version 2.10.2.). For details see ref[6].

3.2 HPLC-QTOF-MS

Frozen and ground samples of approximately 100 mg were weighed in 2.2 ml Eppendorf tubes. Aqueous-methanol extracts were prepared from each RIL and separated using an Alliance 2795 HT system (Waters Corporation) equipped with a Luna C_{18}-reversed phase column (150 x 2.1 mm, 3 µm; Phenomenex, CA). Compounds eluting from the column were detected on-line, first by a Waters 996 photodiode array detector at 200-600 nm and then by a Q-TOF Ultima MS (Waters) with Electron Spray Ionization (ESI) source. Ions were detected in negative mode in the range of m/z 100 to 1500, using a scan time of 900 msec and an inter-scan delay of 100 msec. For details see ref[1].

4. Phenotypic data collected on the same RILs

We defined *phenotypic trait* as the observable morphological, physiological, pathological or biochemical characteristics of an organism as determined by traditional *low-throughput* measurement technologies. Alternative definitions are possible, for example,

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excluding molecular traits such as ion content or enzyme activities. Such reassignments did not have a major impact on our results.

We have collected all publicly available phenotypic and biochemical data on the Ler × Cvi recombinant inbred population from the literature. **Supplementary Table 1** lists all the phenotypic and biochemical traits and gives the corresponding literature reference. Traits can be clustered into more general categories, such as flowering and germination (physiological). **Supplementary Fig. 2** and **Supplementary Fig. 3** show our analysis results for categorized traits.

5. Statistical analysis

5.1 QTL mapping

We performed QTL mapping using two-part multiple-QTL models[7] for transcript, protein, metabolite and phenotypic trait data. The overall false discovery rate (FDR) was set to 0.05. Traits can map to one or more QTLs, and for each QTL the most significant marker was stored for QTL hotspot analysis (see below).

5.2 QTL hotspot analysis

We computed significance thresholds for (i) detection of QTLs hotspots per level, and (ii) detection of hotspots that appear across multiple levels ('system-wide QTL hotspots').

QTL hotspots per level. We used QTL and permutation analysis to compute significance thresholds for detection of QTL hotspots. Redundancy in the biomolecular or phenotypic profiling can lead to correlated traits. In the absence of a common genetic basis, such correlation may still inflate the number of false QTLs at particular loci if trait data are permuted. We therefore permuted marker data to maintain the correlation structure in the trait data. This gives appropriate significance thresholds for detection of QTL hotspots[8]. For each of 250 permutations we analyzed all > 40,000 traits to map QTLs, stored the most significant marker for each QTL, counted the number of significant QTLs over all traits for each marker, stored the maximum value along the genome, and used them to derive significant thresholds for hotspot detection per level (*P* value=0.05).

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System-wide QTL hotspots. We used the observed QTL hotspots and permutation analysis to compute significance thresholds for detection of QTL hotspots that appear at multiple levels. Using the results from the QTL analysis per level, we ranked the markers per level from the one with the highest to that with the lowest number of traits mapping to it. We used a rank-product test[9] to find markers that rank significantly high at multiple levels. For each of 5,000 permutations we computed the *P*-values for the rank-product test at each of the 144 markers, and used them to derive a threshold for hotspot detection at a false discovery rate (FDR) of 5%. QTL hotspots with significant rank-test results (suggesting significant downstream effects) are indicated with arrows above the heat maps (**Fig. 1** in main paper).

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